Noncontact, low-frequency ultrasound as an effective therapy against *Pseudomonas aeruginosa*–infected biofilm wounds

Akhil K. Seth, MD[1]; Khang T. Nguyen, BA[1]; Matthew R. Geringer, BS[1]; Seok J. Hong, PhD[1]; Kai P. Leung, PhD[2]; Thomas A. Mustoe, MD[1]; Robert D. Galiano, MD[1]

1. Division of Plastic Surgery, Feinberg School of Medicine, Northwestern University, Chicago, Illinois, and
2. Microbiology Branch, US Army Dental and Trauma Research Detachment, Institute of Surgical Research, Fort Sam Houston, Texas

Reprint requests:
Dr. R. D. Galiano, Northwestern Plastic Surgery, 675 N. St. Clair, Suite 19-250, Chicago, IL 60611, USA.
Tel: +1 312 695 6022;
Fax: +1 312 695 5672;
Email: rgaliano@nmh.org

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ABSTRACT

Bacterial biofilms, a critical chronic wound mediator, remain difficult to treat. Energy-based devices may potentially improve healing, but with no evidence of efficacy against biofilms. This study evaluates noncontact, low-frequency ultrasound (NLFU) in the treatment of biofilm-infected wounds. Six-millimeter dermal punch wounds in rabbit ears were inoculated with 10^7 colony-forming units of *Pseudomonas aeruginosa* or left as sterile controls. A biofilm was established in vivo using our published model. NLFU treatment was carried out every other day or every day, with contralateral ear wounds acting as internal, untreated controls. Wounds were harvested for several quantitative endpoints and scanning electron microscopy to evaluate the biofilm structure. The *P. aeruginosa* biofilm consistently impaired wound epithelialization and granulation. NLFU, both every other day and every day, improved healing and reduced bacterial counts relative to untreated controls ($p < 0.05$). Scanning electron microscopy confirmed a qualitative decrease in bacteria after both treatments. NLFU also reduced inflammatory cytokine expression ($p < 0.05$). Our study suggests that NLFU is an effective therapy against *P. aeruginosa* wound biofilm. This represents the first in vivo evidence of energy-based modalities' impact on wound biofilm, setting the foundation for future mechanistic studies. Continued wound care technology research is essential to improving our understanding, and treatment, of biofilm-infected chronic wounds.

The effective care of chronic wounds continues to be a difficult, and expensive, problem for clinical practitioners.[1–3] Although several disease processes can contribute to chronic wound pathogenesis, including diabetes mellitus, obesity, and peripheral vascular disease,[4–8] the importance of bacterial biofilms is now being recognized within the scientific community.[9–12] As the predominant state of bacteria within the human body,[13] the biofilm structure provides bacteria with a number of mechanisms for defense and survival against their host's inflammatory response, distinguishing biofilm bacteria from their free-floating, "planktonic" counterparts. The self-secreted extracellular polymeric substance (EPS) that surround bacteria within a biofilm provides a physical barrier to host-derived phagocytosis and complement activation while also preventing the penetration of antibiotics or other externally applied therapeutic agents.[14,15] Biofilms are also dynamic in their ability to utilize protective cell–cell communication, termed quorum sensing, and shed planktonic bacteria in an effort to establish new biofilm populations.[9,10,16] The ultimate outcome is an impairment of wound healing, now shown in several in vitro, in vivo, and clinical models.[11,17–21]

The durability of a biofilm and its significance to chronic wound healing underscore the need for an evolution in current wound care therapy. Wound-bed preparation and treatment have traditionally centered around therapies such as debridement, lavage, and antimicrobials, but with little evidence that they improve chronic wound healing in a quantitative and consistent manner.[22,23] Although we have recently demonstrated that frequent and aggressive, multimodal therapies may be effective in reducing wound biofilm,[20] treatment regimens specifically aimed at biofilm development and maintenance are limited and unproven. Molecular therapies, such as the introduction of D-amino acids[24,25] and RNA-inhibiting peptides,[18] have shown some efficacy both in vitro and in vivo, but the translation of these modalities to the clinical setting remains prohibitive. Meanwhile, protocols involving the use of specialized dressings have been tested in several different settings, but with mixed efficacy.[26,27] Despite a growing understanding of the mechanisms underlying biofilm virulence, the application of this knowledge toward developing effective, antibiofilm therapies has not progressed as rapidly.

In an effort to diversify the current wound care modalities against biofilms, energy-based treatment devices have emerged as potential alternatives to the aforementioned traditional methods. In particular, noncontact, low-frequency ultrasound (NLFU) therapy has recently been shown to improve wound healing in a variety of clinical settings, including in diabetic and ischemia-based ulcers and in burns.[28–34] Other studies have shown a reduction in bacterial burden[35–37] and the stimulation of host inflammatory and

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wound healing pathways secondary to ultrasound exposure.[38] The coupling of the low-frequency, ultrasonic waves with saline allows for the translation of the generated energy to the wound bed.[36] However, despite the growing literature, the efficacy of this therapy in the setting of established bacterial biofilms remains unclear.

The goal of this study was to evaluate the efficacy of NLFU against wound biofilm using an established, in vivo, rabbit ear model. Utilizing a Food and Drug Administration–cleared, ultrasound delivery device (MIST Therapy System; Celleration Inc., Eden Prairie, MN), we have demonstrated a qualitative and quantitative reduction in biofilm burden, with subsequent improvements in host wound healing and inflammatory dynamics. Furthermore, using results from the treatment of uninfected, rabbit ear wounds as a control, we have provided evidence to support a specific role for NLFU therapy in treating biofilm-infected, chronic wounds.

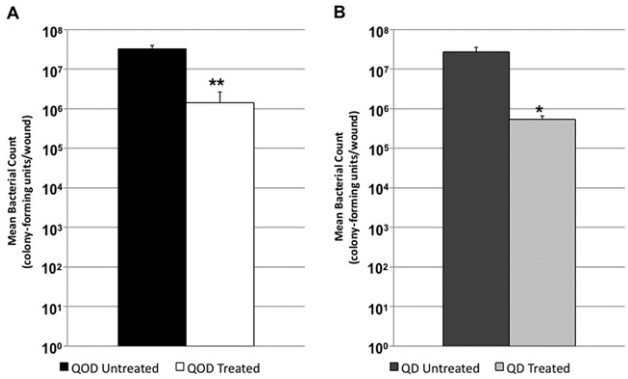

Figure 1. Viable bacterial counts from *Pseudomonas aeruginosa*–infected biofilm wounds with and without NLFU treatment. Wounds treated QOD (A) and QD (B) showed significant reductions in viable bacteria at POD 12 relative to untreated, control wounds. $*p < 0.05$, $**p < 0.01$. $n = 6$ wounds/group. NLFU, noncontact, low-frequency ultrasound.

METHODS

Animals

Under an approved protocol by the Animal Care and Use Committee at Northwestern University, adult New Zealand white rabbits (3–6 months, ~3 kg) (Covance, Princeton, NJ) were acclimated to standard housing and fed ad libitum. All animals were housed in individual cages under constant temperature and humidity, with a 12-hour light : dark cycle. A total of 13 animals were used for this study.

Bacterial strains and culture

Wild-type strain of *Pseudomonas aeruginosa* PAO1 (obtained from the laboratory of Dr. Barbara H. Iglewski, University of Rochester Medical Center) was utilized for wound infection. *P. aeruginosa* was grown overnight at 37 °C on *Pseudomonas* Isolation Agar (Hardy Diagnostics, Santa Maria, CA), and co-cultured in Luria broth, at 37 °C until log-phase was achieved. Bacteria were harvested and washed in phosphate-buffered saline (PBS) three times by centrifugation at 978.25 *g* for 5 minutes at 20 °C. An optical density at the 600-nm wavelength (OD_{600}) was measured and bacterial solution diluted to match an OD_{600} equivalent to 10^6 colony-forming units (CFUs)/μL, which was empirically predetermined.

Wound protocol and infection model

Wounding, bacterial infection, and biofilm formation were adapted from principles established in our previously published in vivo, wound biofilm model.[18] Rabbits were anesthetized with intramuscular injection of a ketamine (22.5 mg/kg) and xylazine (3.5 mg/kg) mixture prior to surgery. Ears were shaved, sterilized with 70% ethanol, and intradermally injected with a 1% lidocaine/1 : 100,000 epinephrine solution at the planned wound sites. Six 6-mm-diameter, full-thickness dermal wounds were created on the ventral ear down to the perichondrium and dressed with Tegaderm (3M Health Care, St. Paul, MN), a semi-occlusive transparent film. Individual biofilm wounds were inoculated with *P. aeruginosa* on postoperative day (POD) 3. Bacterial solutions were diluted such that each wound was inoculated with a total of 10^7 CFU of bacteria at a volume of 10 μL. Bacteria were allowed to proliferate in vivo under the Tegaderm dressing. Topical antibiotics (Ciloxan ointment [Ciprofloxacin 0.3%, Alcon, Fort Worth, TX]) were applied on POD 4 to eliminate free-floating, planktonic-phase bacteria, leaving a predominantly biofilm-phase phenotype. To prevent seroma formation and regrowth of planktonic bacteria, thus maintaining a biofilm-dominant infection, an antimicrobial, absorbent dressing containing polyhexamethylene biguanide (Telfa AMD, Tyco Healthcare Group, Mansfield, MA) was applied to biofilm wounds on PODs 5 and 6 and then every other day (QOD) until harvest. For clean wounds, a similar wounding and dressing change protocol was followed, but without bacterial inoculation or antibiotic application. All dressings were checked daily (QD) throughout the protocol.

Study design and treatment protocol

Animals were designated to one of two experimental study arms: biofilm wounds ($n = 9$ animals) and clean wounds ($n = 4$ animals). For each rabbit, ears were then designated as either the "untreated" or the "treated" ear, with each of the six wounds on that ear following the same protocol. This allowed for each wound to have its own internal control on the contralateral ear for improved statistical validity. NLFU treatment of wounds on treated ears was carried out using the MIST Therapy System (Celleration, Inc.) (Figure 1). Treatments were administered to *P. aeruginosa* biofilm-infected wounds either QOD or QD starting on POD 6, the time at which a steady-state, predominantly biofilm infection is present.[18,19] After each treatment, new Telfa and Tegaderm dressings were reapplied. Similarly, clean wounds underwent treatment QOD starting on POD 6. A standardized treatment time of 3 minutes was used for each wound. This was dictated by the algorithm relating treatment time to wound size, as stated in the "Instructions for Use" provided with the device. On POD 12, after euthanizing the animals by intracardiac euthasol injection, wounds were harvested for various analyses. All wounds

were excised using a 10-mm (viable bacterial counts, histology, scanning electron microscopy [SEM]) or a 7-mm (quantitative-reverse transcription-polymerase chain reaction [Q-RT-PCR]) biopsy punch (Acuderm Inc., Fort Lauderdale, FL).

Viable bacterial count measurements

The dorsal sides of wounds used for bacterial counts were removed to eliminate the inclusion of bacteria outside of the infected wound surface. To recover bacteria, *P. aeruginosa*-infected biofilm wound samples were placed in tubes prefilled with homogenizer beads (Roche, Indianapolis, IN). One milliliter of PBS was added to the tube and was homogenized for 90 seconds at 978.25 *g* in a MagNA Lyser homogenizer (Roche Diagnostics), followed by sonication (Microson Ultrasonic Cell Disruptor, Heat Systems Ultrasonics, Inc., Farmingdale, NY) for 2 minutes at 6–8 W to disrupt any biofilm present. The resulting solutions were serially diluted and plated on *Pseudomonas* Isolation Agar plates and incubated overnight at 37 °C. CFUs were determined by a standard colony-counting method.

Histological analysis

Wounds excised for histological analysis were bisected at their largest diameter for hematoxylin & eosin (H&E) staining. Tissues were fixed in formalin, embedded in paraffin, and cut into 4-μm sections. Paraffin was removed with a xylene wash, followed by a standard H&E staining protocol to prepare samples for analysis under a light microscope. Slides were examined for quantification of epithelial and granulation gaps, and total epithelial and granulation areas, using a digital analysis system (NIS-Elements Basic Research, Nikon Instech Co., Kanagawa, Japan), as previously described.[18–20] Two blinded, independent observers evaluated all histological sections, and the results of both examiners were averaged.

SEM

To visualize biofilm structure, wound samples were fixed in 2.5% glutaraldehyde in 0.1-M PBS (pH 7.2), washed three times in PBS, and dehydrated through an ethanol series and hexamethyldisilazane. Samples were mounted by a double-sided tape to specimen stubs, followed by gold–platinum (50 : 50) ion coating (108 Auto Sputter Coater, Ted Pella, Inc., Redding, CA). Wounds for SEM had their dorsal sides removed prior to preparation to allow for better mounting for visualization. Samples were visualized using a Carl Zeiss EVO-40 SEM (Carl Zeiss, Oberkochen, Germany) operated at the scanning voltage of 10 kV.

Total mRNA extraction and Q-RT-PCR

Wounds were harvested for mRNA extraction and subsequent cDNA conversion as part of Q-RT-PCR. The dermal layer on the dorsal side of the ear was removed, and the wound bed was punched out and immediately snap-frozen in liquid nitrogen. Wound samples were homogenized using a Mini-bead beater-8 equipment (Biospec Products Inc., Bartlesville, OK) using Zirconia beads (2.0-mm diameter, Biospec Products Inc.) in the presence of TRIzol Reagent (Sigma-Aldrich, St. Louis, MO). Total RNA was isolated according to the manufacturer's protocol. Contaminating genomic DNA during RNA preparation was removed using the Turbo DNA-free kit (Ambion, Austin, TX). Five micrograms of total RNA was used to prepare cDNA using superscript II (Invitrogen, Carlsbad, CA) with 100 ng of random primers (Invitrogen).

For quantitative analysis of the level of mRNAs, Q-RT-PCR analyses, using SYBR Green I, were carried out utilizing an ABI prism 7000 sequence detection system (Applied Biosystems, Foster City, CA). PCR primers were designed using the Primer 3 program (http://frodo.wi.mit.edu/). Expression of each gene was normalized to the level of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), the housekeeping gene, to get the ΔCt. The $2^{-\Delta\Delta Ct}$ method was used to calculate the gene expression of interleukin-1-beta (IL-Iβ) and tumor necrosis factor-α (TNF-α) within the wounds of interest. The expression of genes was detected by PCR with the following oligonucleotides: IL-Iβ (5′-CCACAGTG GCAATGAAAATG-3′ and 5′-AGAAAGTTCTCAGGCCGT CA-3′, accession number D21835), TNF-α (5′- CCAGATGG TCACCCTCAGAT-3′ and 5′-tgttctgagaggcgtgattg-3′, accession number M12845), GAPDH (5′-aggtcatccacgaccacttc-3′ and 5′-gtgagtttcccgttcagctc-3′, accession number NM_ 001082253).

Statistical analysis

Data were presented in graphical form as mean ± standard errors when applicable. Statistical analyses were carried out using a paired, two-tailed Student's *t* test with the comparison of each treated wound with its paired, untreated control. The level of significance was set at $p < 0.05$. All analyses were carried out at Northwestern University.

RESULTS

Using our previously established model of in vivo, *P. aeruginosa* wound biofilm,[19] biofilm-infected wounds were treated using NLFU and compared with untreated, internal control wounds. For both QOD and QD treatment frequencies, NLFU resulted in a significant reduction in viable bacterial counts relative to control (QOD: $p < 0.01$; QD: $p < 0.05$) (Figure 1). This reduction was almost 2-log-fold for both treatment regimens. This improvement in bacterial burden was visualized on the SEM (Figure 2). Untreated control wounds showed relatively large amounts of rod-shaped bacteria with a dense extracellular matrix between individual cells (Figure 2A). In comparison, wounds treated QOD (Figure 2B) and QD (Figure 2C) treated wounds revealed large areas of bare wound bed (arrows) with less overall bacteria. This was confirmed at higher magnification in QOD-treated wounds (Figure 2D), along with the presence of relatively less extracellular matrix compared with the control wounds.

The reduction in bacteria counts secondary to NLFU correlated with a simultaneous improvement in wound healing in QOD- and in QD-treated wounds at POD 12. These improvements could be seen histologically (Figure 3), with QOD (Figure 3B) and QD (Figure 3C) wounds showing increased granulation tissue and decreased epithelial and granulation gaps relative to control wounds (Figure 3A). These visual trends were validated through quantification and analysis of

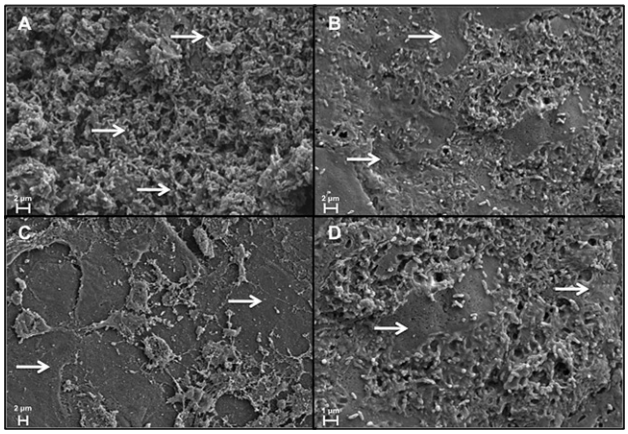

Figure 2. Scanning electron microscopy of *Pseudomonas aeruginosa*–infected biofilm wounds with and without NLFU treatment at POD 12. Untreated wounds (A) reveal large amounts of rod-shaped bacteria (arrows) with interspersed extracellular matrix. In contrast, QOD-treated (B) and QD-treated (C) wounds show significantly reduced amount of bacteria and associated matrix, revealing areas of bare wound bed (arrows), which are infrequent in untreated wounds (A). Higher magnification of QOD-treated wounds verifies the presence of only small amounts of bacteria with multiple areas of clean wound bed (D) (arrows). NLFU, noncontact, low-frequency ultrasound.

our standard histological parameters (Figure 4). QOD-treated wounds showed significant improvements in epithelial ($p < 0.001$) and granulation ($p < 0.001$) gaps, as well as new epithelial ($p < 0.01$) and granulation ($p < 0.05$) tissue areas relative to control wounds (Figure 4A and B). Similar trends were seen on comparison of the QD-treated and control wounds, although the improvements were not as statistically significant ($p < 0.05$) (Figure 4C and D). However, on comparison of both treatment cohorts, there were no differences in any of our measured histological parameters between the two groups, indicating a level of equivalency between QOD- and QD-treatment frequencies for NLFU therapy in our model (Figure 5). Analysis of cytokine mRNA levels using Q-RT-PCR revealed additional improvements in the host inflammatory response in NLFU-treated wounds (Figure 6). QOD treatment of *P. aeruginosa*–infected biofilm wounds resulted in a significant reduction in IL-Iβ ($p < 0.01$) and TNF-α ($p < 0.05$) mRNA levels at POD 12 relative to untreated, infected control wounds.

Having shown a significant reduction in *P. aeruginosa* biofilm burden secondary to NLFU therapy, with a corresponding change in inflammatory response and wound healing, additional control experiments using uninfected wounds were carried out in an attempt to understand the mechanisms behind these findings. With no significant differences seen previously between QOD and QD treatment frequencies, QOD treatment was carried out on clean, uninfected wounds, with comparison to untreated, internal controls. Interestingly, on histological imaging, NLFU-treated wounds showed an increase in the granulation tissue area (untreated: 279.8 mm^2; treated: 336.4 mm^2; $p < 0.05$) but no difference in the granulation gap compared with the untreated wounds (Figures 7 and 8). In both groups, the majority of the wounds were epithelialized (untreated: 88.9% [16/18]; treated: 83.3% [15/18]; $p = 1.00$). In general, the epithelialization is so brisk in normal wound healing such that it is possible an improvement in this parameter would have been seen if compromised wounds (e.g., ischemic, diabetic, or radiated) were looked at. Similarly, the treatment of uninfected wounds did not result in a reduction in inflammatory cytokine mRNA levels (Figure 9). However, expression levels in both untreated and treated wounds were found to both be close to the baseline values seen in nonwounded skin (level of mRNA fold difference = 1) for both cytokines.

DISCUSSION

The pathogenesis of chronic wounds remains complex and multifactorial.[1–8] As a critical contributor to chronic wound healing impairment, the robust defense mechanisms and durability of bacterial biofilms have made the treatment of these wounds much more challenging.[9–21] With continued research aimed at several different potentially therapeutic modalities, NLFU has emerged as having clinical efficacy for chronic wound care, but to date not specifically against established wound biofilm.[28–38] Using a published in vivo, rabbit ear, wound biofilm model, we have demonstrated that NLFU effectively decreases the viable bacterial burden of *P. aeruginosa*–infected biofilm wounds. This results in both significantly improved wound healing and a decreased host inflammatory response relative to untreated wounds.

To date, only a paucity of literature has addressed the treatment of wound biofilm using energy-based modalities.

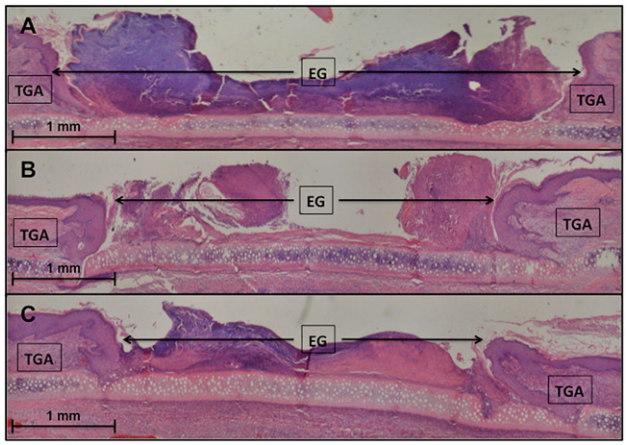

Figure 3. Histological sections of untreated and NLFU-treated, *Pseudomonas aeruginosa*–infected, biofilm wounds stained with hematoxylin & eosin at POD 12. Untreated control wounds (A) show decreased amounts of new epithelial and granulation tissue than QOD-treated (B) and QD-treated (C) wounds, including a larger EG and a smaller amount of TGA. However, comparison of wounds with different treatment frequencies reveals similar amounts of healing between the two groups. EG, epithelial gap; NLFU, noncontact, low-frequency ultrasound; TGA, total granulation area. Magnification ×20.

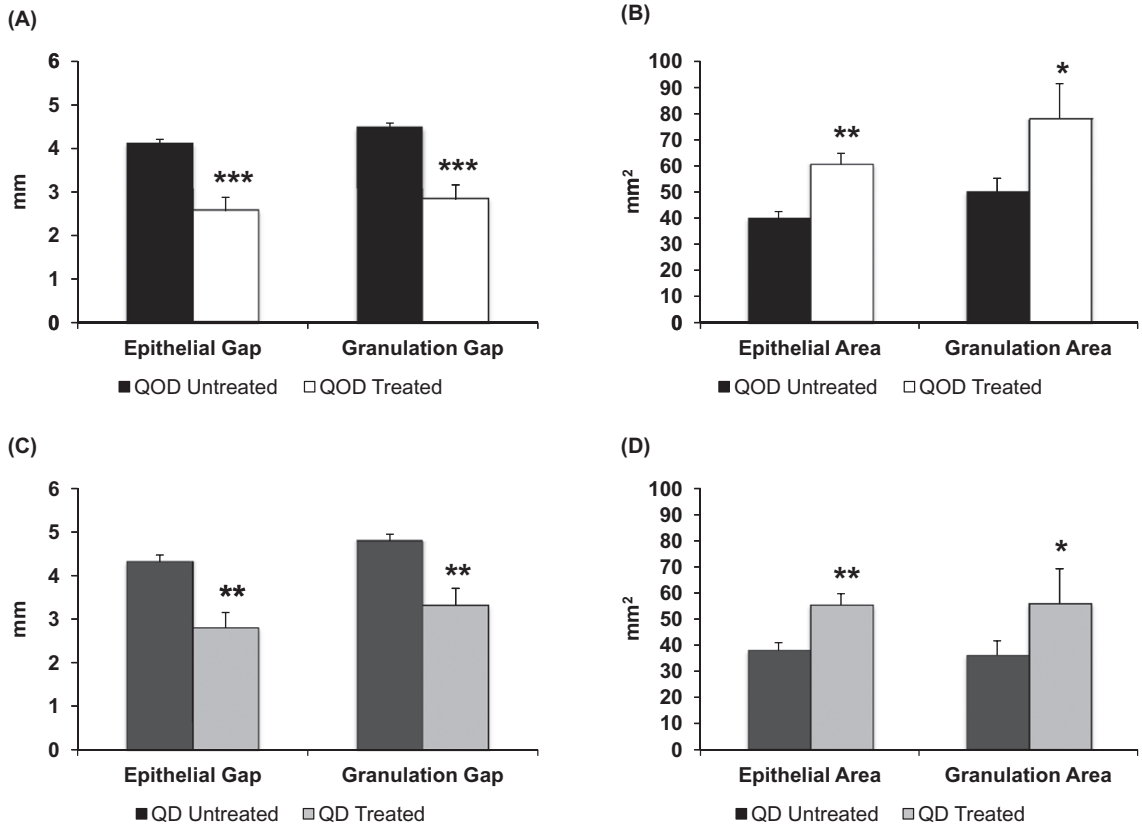

Figure 4. Comparison of quantitative histological parameters between untreated control and the NLFU-treated *Pseudomonas aeruginosa*–infected biofilm wounds. At both QOD (A and B) and QD (C and D) treatment frequencies, NLFU therapy resulted in significant improvements in all measured healing parameters, including smaller epithelial and granulation gaps, and increased epithelial and granulation tissue areas. $*p < 0.05$; $**p < 0.01$; $***p < 0.001$. $n = 15$ wounds/group. NLFU, noncontact, low-frequency ultrasound.

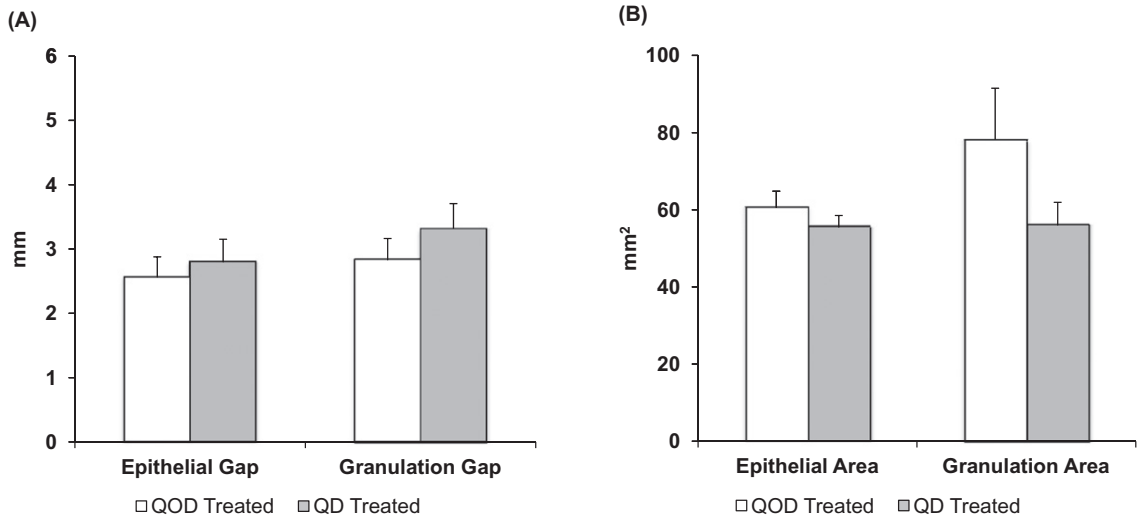

Figure 5. Comparison of quantitative histological parameters between NLFU-treated, *Pseudomonas aeruginosa*–infected, biofilm wounds at QOD and QD frequency. For all measured histological parameters, there were no significant differences between wounds treated with NLFU therapy QOD or QD. $n = 15$ wounds/group. NLFU, noncontact, low-frequency ultrasound.

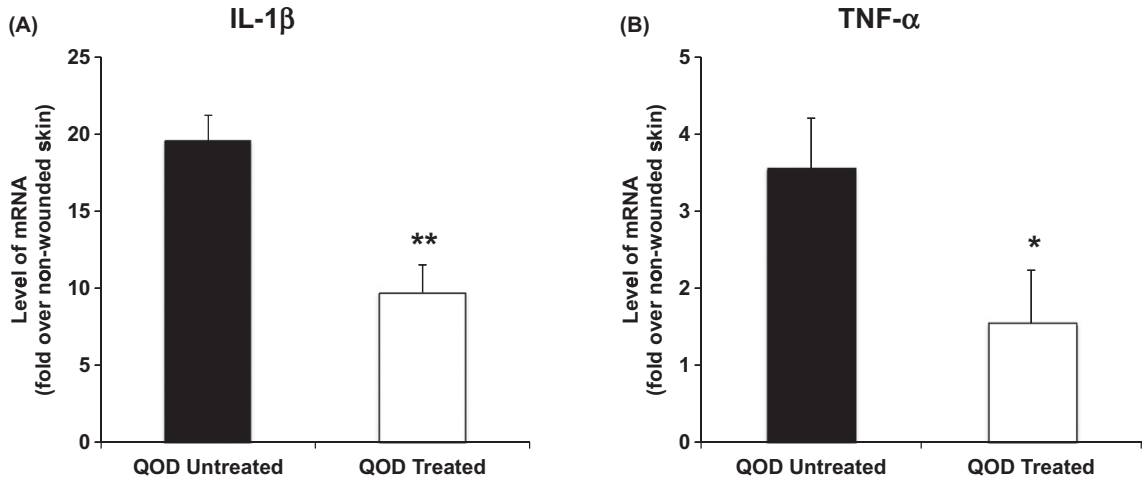

Figure 6. Inflammatory cytokine mRNA levels in *Pseudomonas aeruginosa*–biofilm-infected wounds with and without NLFU treatment. Wounds treated with NLFU QOD showed reductions in host inflammatory response relative to untreated, biofilm infected wounds, represented by significant decreases in mRNA levels of IL-Iβ (A) and TNF-α (B). $*p < 0.05$; $**p < 0.01$. $n = 6$ wounds/group. NLFU, noncontact, low-frequency ultrasound.

Work by Street et al.,[39] using photodynamic disinfection technology, has shown the eradication of in vitro planktonic and biofilm cultures of *P. aeruginosa*, which, as they have suggested, may ultimately be a clinical alternative to antibiotics for infected wound treatment. Meanwhile, with regard to NLFU, Thawer and Houghton[40] demonstrated increased deposition of collagen and blood vessels in an in vivo diabetic mouse model but did not utilize a bacterial challenge as part of their experiments. Several other clinical studies have assessed the efficacy of NLFU against chronic wounds, including a clinical trial[29] and a randomized, double-blinded, multicenter trial[28] by Ennis et al., demonstrating improvements in healing rates without adverse events in both cases. Studies by Kavros et al[30,31] have confirmed these findings, commenting that NLFU was particularly effective when combined with other standard wound care methods, including QD dressing changes and wound debridement. The mechanism for the observed improvement in healing was not examined. In addition, unfortunately, none of these clinical studies had established the presence of bacterial biofilm within these clinical wounds prior to treatment. Waldrop and Serfass[32] also showed that ultrasound treatment could be an effective adjunct to conventional burn care in a small case series, although again without including wounds with a baseline level of bacterial burden.

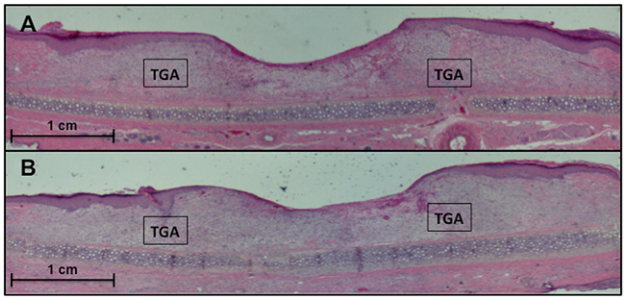

Figure 7. Histological sections of uninfected, control wounds with and without NLFU treatment, stained with hematoxylin & eosin at POD 12. QOD treatment of the uninfected, clean control wounds (B) did not appear to improve histological wound healing over untreated, clean control wounds (A). Both wounds show complete epithelialization, with similar amounts of a new TGA. NLFU, noncontact, low-frequency ultrasound; TGA, total granulation tissue area. Magnification ×20.

The data presented in our study suggest that NLFU has a significant impact on biofilm-infected wounds, which is specific to biofilm itself. This includes both a decrease in viable bacteria, as well as an overall improvement in wound healing and host inflammatory dynamics. Although the mechanisms underlying these benefits are not immediately clear, our findings allow for speculation as to why this treatment modality may be effective. Previous studies have shown physical effects on cells and their surrounding matrix due to ultrasound energy, termed cavitation and microstreaming.[29] This energy may also have a disruptive effect on the intricate extracellular structure of biofilm, which is responsible for a number of its survival and defense mechanisms. In particular, the EPS for *P. aeruginosa* appears to be critical to its virulence, based on previous work using this model.[41] With an ineffective EPS, host inflammatory cells may be able to effectively penetrate the wound biofilm to eliminate resident bacteria, triggering subsequent improvements in wound healing while minimizing the counterproductive chronic inflammatory response characteristic of the biofilm phenotype. In addition, albeit less powerful than traditional water lavage, the saline mist that is coupled to the delivered ultrasound energy may also provide some mechanical benefit against bacteria, particularly if they lack a protective extracellular matrix.

Although a proposed mechanism for NLFU efficacy is its ability to stimulate host cell pathways,[35] in turn improving wound healing, our results indicated no major differences between treated and untreated wounds that were not infected

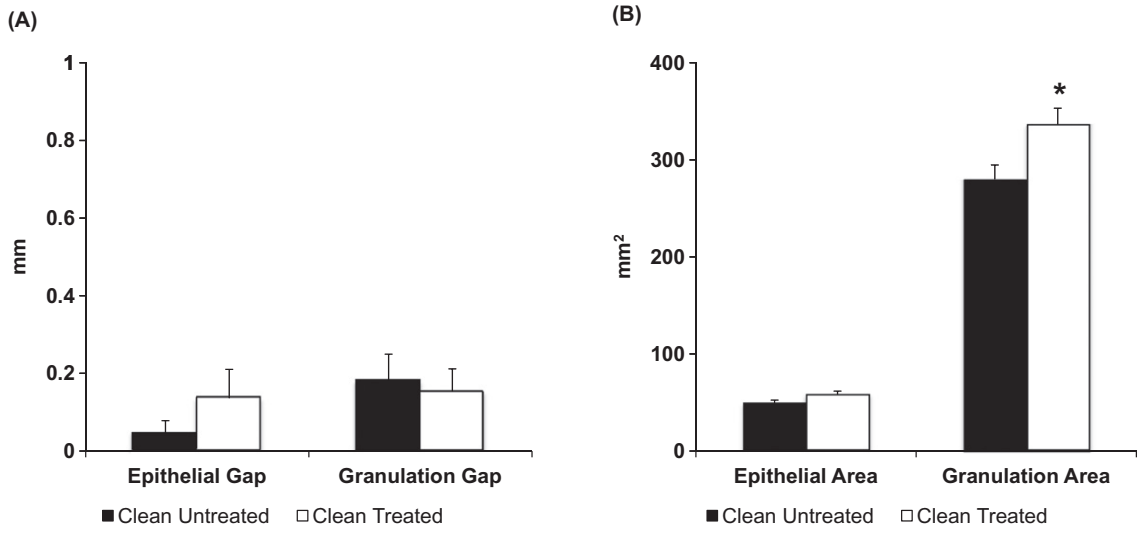

Figure 8. Comparison of quantitative histological parameters between untreated and NLFU-treated uninfected, clean control wounds. NLFU treatment QOD did not decrease the epithelial or granulation gap (A) or increase the epithelial area (B) relative to untreated, clean control wounds at POD 12. However, NLFU treatment resulted in a significant improvement in a new granulation tissue area. * $p < 0.05$. $n = 18$ wounds/group. NLFU, noncontact, low-frequency ultrasound.

with biofilm. However, it is important to recognize that the time line used for our biofilm wounds was not designed for the dedicated study of normal wound healing, unlike in previous work that has shown improvements with NLFU.[28–34] We did demonstrate a significant increase in the granulation tissue area, indicating that the use of compromised wounds, which are more sensitive and have greater room for improvement, may have resulted in larger differences in healing following treatment. Nevertheless, the goal of this study was to understand the efficacy of NLFU against wound biofilm, for which these wounds served as an appropriate control.

Interestingly, the frequency of NLFU therapy, QOD or QD, within our in vivo biofilm model did not affect our end results, specifically bacterial burden and wound healing. Previous work with *P. aeruginosa*–infected biofilm wounds by our group has shown that the use of traditional wound care treatment modalities, such as debridement, lavage, and topical antibiotics, can be effective in the face of biofilm when used

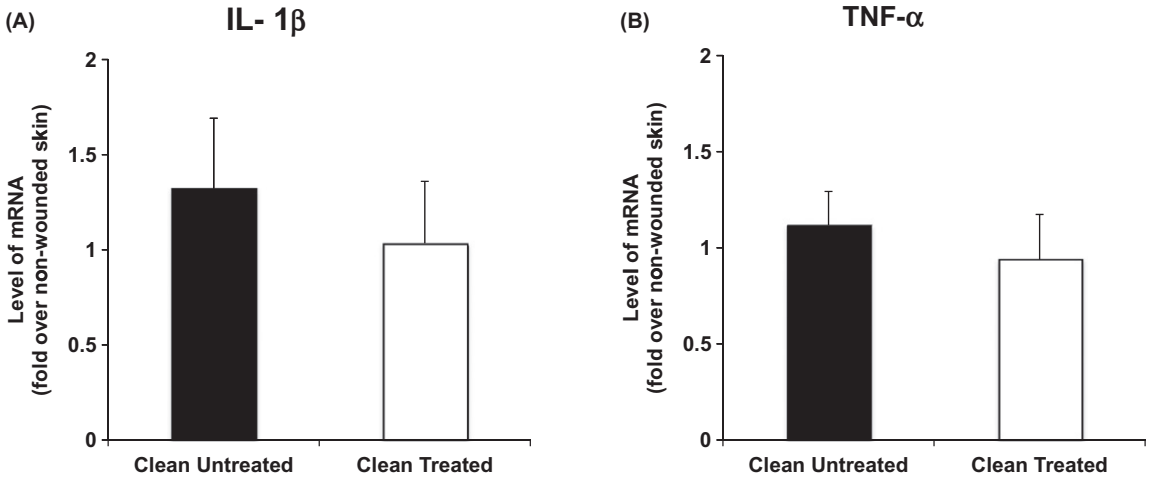

Figure 9. Inflammatory cytokine mRNA levels in uninfected, clean control wounds with and without NLFU treatment. QOD NLFU treatment did not result in a decrease in mRNA levels of inflammatory cytokines IL-Iβ (A) and TNF-α (B) relative to untreated, clean control wounds. As seen graphically, for both groups, the level of mRNA for untreated and treated wounds appeared to be close to that of the nonwounded skin, resulting in a ratio of close to 1. $n = 6$ wounds/group. NLFU, noncontact, low-frequency ultrasound.

in combination and/or with increased frequency.[33] However, these principles appear to stand in contrast to our current findings. With unclear mechanisms underlying ultrasound wound therapy, it is possible that the cells within the host wound bed may show a "refractory period," with the end effect of a treatment plateauing before a second treatment can have a significant impact. Correlating with this potential theory is the reported efficacy of clinical NLFU when treatments are carried out at least 24–72 hours apart.[28–31,33] However, Kavros and Schenck[33] have suggested that increasing the frequency of treatments may be beneficial based on their retrospective clinical analysis. Although our study may be underpowered to make a true determination of a potential difference, consideration should be given to the temporal nature of NLFU efficacy in future studies aimed at identifying its mechanism of action.

The promising findings that we have presented cannot be discussed without acknowledging the limitations of our study. In particular, we utilized low-frequency ultrasound against only one bacterial species, despite several different species of bacteria often being within the same chronic wound.[20] Future studies aimed at species such as *Staphylococcus aureus* or at in vivo polybacterial biofilms will further our understanding of NLFU's efficacy. Similarly, we also did not combine NLFU treatment with any other more traditional therapies to determine the effects of a multimodal approach. However, without any prior experience using NLFU in our model, we hoped to establish whether or not the therapy had a baseline level of efficacy against biofilms prior to potentially enhancing this benefit with modalities such as debridement or antibiotics.

With a continued need for innovation within the field of wound care, the recognition and testing of new therapeutic modalities is critical. In particular, the robustness of wound biofilm warrants the development of new, nonantimicrobial-based technologies that specifically target a biofilm's fundamental structure and mechanisms. With growing evidence in support of its efficacy in chronic wound care, NLFU appears to be particularly effective in the setting of infected chronic wounds. Using our results as a foundation, we believe further iterations with this and other new technologies will contribute toward a new revolution in wound care, ultimately expanding the armamentarium of practicing wound care clinicians.

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